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Article

Antimicrobial activity of citrus semivolatile compounds obtained through a new enzyme assisted extraction technique

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Abstract
A continuous population and industrialization growth have enhanced the production of chemical compounds as well as their structural chemical diversification. Unfortunately, they have become an increasing burden for the environmental sustainability and biodiversity. The need for producing and using environmentally friendly products become increasingly evident in the current context of environmental concerns. It is crucial to develop new products and technologies that reduce their overall harmful environmental impact and promote its sustainability. In this study, the citrus peels waste was transformed into a potential antibacterial and antifungal extract by hydrodistillation. The efficacy of orange peels hydro distillation process in obtaining plant extracts was improved by a pre-enzyme-assisted extraction step. Enzymes such as cellulase and pectinase improved the breakdown of cellulose and pectin structures, which led to an increased extraction yield of bioactive compounds and antimicrobial activity.

Keywords: *antimicrobial, citrus peels, ecofriendly, enzyme-assisted extraction hydrodistillation*

INTRODUCTION

Herbs and herbal medicines have a long history, dating back to ancient times. Overtime, plant extracts, referred as moist or dry pharmaceutical/phyto-pharmaceutical products, improved their beneficial effect by various extraction processes using different solvents. Plant compounds such as proteins, carbohydrates or amino acids are main plant metabolites involved in plant development and maturation. Secondary metabolism process occurs during the developmental cycle to assist plants in surviving and overcoming natural obstacles. Plant metabolites such as bioactive compounds may be found in a wide range of plant articles (leaves, flowers, root) being categorized into several classes of compounds like terpenoids, alkaloids, compounds containing nitrogen, compounds with sulfur atoms, and phenolic compounds. Even though a plant extract may have thousands of distinct secondary metabolites, a phytochemical study will only identify a small portion of its components [1, 2]. According to Statista 48 million metric tons of orange was produced in 2022/2023 [3]. Orange peels are abundant in vitamins (such as vitamin C), minerals, and dietary fiber. Additionally, they contain bioactive compounds with demonstrated antioxidant, anti-inflammatory, and antimicrobial properties [4]. A wide range of applications derived from orange peel antimicrobial extracts with environmental benefits such as waste reduction and environmental ecofriendly products [5, 6]. Orange peels are frequently discarded as waste, contributing to environmental pollution, but repurposing them reduces the volume of organic waste. This practice aligns with principles of circular economy and sustainable development by converting waste into valuable products [7, 8]. Economic advantages are also notable, especially by extracting valuable compounds from orange peels that

could generate additional revenue streams for agriculture and food industries. Moreover, utilizing a byproduct (orange peel) rather than cultivating or sourcing new raw materials can lower production costs [9]. Health benefits are derived from the antimicrobial properties of orange peel. Compounds such as flavonoids, essential oils, and phenolic acids have been shown to inhibit the growth of harmful bacteria and fungi. These orange peel extracts can be used in food preservation, pharmaceuticals, and cosmetics [10, 11]. For instance, orange peel extracts serve as natural preservatives in food products, extending shelf life and ensuring safety. In pharmaceuticals, these antimicrobial properties can aid in the development of new antibiotics or antiseptics, especially crucial in the context of rising antibiotic resistance [12]. In cosmetics, the antimicrobial and antioxidant properties of orange peel extracts contribute to a healthier skin [13]. At the present, the ,,advanced" extraction techniques outperform traditional ones and therefore obtain high-quality herbal product as bioactive compounds from medicinal plants.

One of the most crucial steps in the production of active herbal compounds is the extraction process, which has a qualitatively and quantitatively impact on plant extractions. Enzyme-based extraction is a substitute of conventional solvent-based extraction methods. Enzymes are ideal catalysts when it comes to enhance the extraction or to modify the synthesis of complex bioactive molecules.

Enzymes can be more efficient in increasing the availability of plant bioactive compounds for extraction, due to their ability to degrade or destroy cell walls and membranes. Plant material is pretreated with a wide range of enzymes such as protease, pectinase, pectinesterase, cellulase, hemicellulase, cellobiase, α-amylase, and fructosyltransferase to release phytochemicals that are linked to lipid and carbohydrate chains inside the cell and break down cell walls. Enzyme-assisted extraction is influenced by many parameters such as enzyme type and concentration or time and temperature of the enzymatic treatment. The structural properties of a plant material such as its chemical makeup, water content, and particle size; and the ratio of solvent to solid play also a role in modulating the enzyme-assisted extraction process [14÷17].

In this study, the antimicrobial effect of orange peel extract was analyzed in the presence of gram-positive bacterial strains. Enzyme-assisted extraction seemed to enhance the quantity and quality of orange extracts which had a superior antibacterial efficiency compared to standard extraction.

MATERIALS AND METHODS

The vegetal material was represented by orange peel. The oranges were collected from the local supermarket of Bucharest (Romania).

Chemical reagents

For preparing enzyme solutions, were used cellulose from *Aspergillus niger* with concentration of 17000 unit/g and pectinase from *Aspergillus niger* with concentration of 300,000 unit/g, purchased from Tokyo Chemical Industry CO., LTD. Japan.

Sodium acetate anhydrous with purity percentage of 99÷100%, and acetic acid anhydrous used for buffer preparation were purchased from Honeywell, Germany.

Tryptic Soy (CASO) Broth Casein-peptone Soymeal-peptone Broth, universal culture media free from inhibitors and indicators for a wide spectrum of applications and utilized for isolation and cultivation of the bacterial strain *Corynebacterium kroppenstedii* was purchased from VWR International Europe, Belgium.

Enzymatic treatment

10 g chopped orange peels were added to 100 mL buffer (0.1 M solution of sodium acetate and acetic acid), then pre-incubated or not (control) in presence of various enzymes at 40ºC for 1h. The enzymatic pretreatment step was followed by the transfer of orange peel-enzyme mixt to a distillation vessel for hydrodistillation under water vapors.

Bacterial strain inhibition screening

The antimicrobial effect of hydrodistilled orange peels was analyzed on the bacterial strain *Corynebacterium kroppenstedii* (*C. kroppenstedii*). The bacterium was identified, isolated and then cultivated in peptone water (AP) medium. The bacterium comes from real aeromicroflora environmental samples taken from the outskirts of Bucharest, in the western part, in two consecutive days. Aeromicroflora samples were taken with SAS AIR SAMPLE (volume 1000 L, 10 minutes) on the CASO nutrient medium and incubated for 48 h at 37ºC. The identifications of the same bacteria provided by the developed cultures was performed with the Omnilog system. Pure bacterial cultures were harvested from the plates obtained and transferred into a liquid medium of AP, which represented the stock solution of bacteria. The degree of growth of the bacterial cultures, was analyzed according to the optical density observed at 600 nm. *C. kroppenstedii* grown in AP was incubated or not (control) in presence hydrodistilled oranges peels at 37ºC up to 2 hours. Bacterial growth was monitored using a spectrophotometer at a wavelength of 600 nm.

Chromatographic technique

The analysis of semi-volatile compounds of peel extracts were performed with the Trace 1310 Thermo Gas Chromatograph equipment coupled with the TSQ 8000 EVO Thermo Mass Spectrometer. Liquid-liquid extraction was performed, for 1 mL sample with 0.4 mL pentane. The separation was carried out with a 5% phenyl, 95% dimethylpolysiloxane capillary column, 60 m long, 0.25 mm internal diameter and 0.25 μm stationary phase thickness. Temperature program: 50°C (1 minute), heating up to 300°C with 10°C/min, 300°C (19min). Injector temperature: 280°C. Sample division ratio:5. Injected volume: 1 μL. Table area used: 50-50 uam. Transfer line temperature: 280°C. Ion source temperature: 250°C.

RESULTS AND DISCUSSION

The objective of the study was to determine if fruit peel extracts enhanced by enzymatic pretreatment have a better antibacterial effect, influencing the potentially pathogenic bacterium, *Corynebacterium kroppenstedtii* growth, compared to those without enzymatic pretreatment.

Samples pretreated with cellulase, pectinase, and a 1:1 mixture of both enzymes were analyzed to identify the most effective formulation for hydrodistillation. The purpose of this pretreatment was to enhance the extraction of volatile compounds, phenolic compounds, hydrophilic and hydrophobic pigments, and other bioactive constituents.

Antibacterial activity of orange peels

The extracts from orange peels obtained through hydrodistillation with enzymatic pretreatment were analyzed measuring optical density at a wavelength of 600 nm. The obtained results are presented in table 1.

Table 1. The optical density of the bacterial culture at 600 nm before and after exposure to plant extracts

Samples	T0	T1h	T2h	Inhibition %, 2h
Control (B in growth medium)	0.347	0.542	1.062	-
Orange peel + B	0.334	0.637	1.175	0
Orange peel +C+B	0.327	0.312	0.481	54%
Orange peel +P+B	0.384	0.421	0.472	55%
Orange peel +C+P+B	0.377	0.348	0.466	56%

Notes:

B: bacterial strain; Orange peel: orange peel without enzymatical pretreatment; Orange peel + B: bacteria incubated in presence of orange peel hydrodistilate; Orange peel+C+B: bacteria incubated in presence of cellulase (C) orange peel pretreated hydrodistilate; Orange peel+P+B: bacteria incubated in presence of pectinase (P) orange peel pretreated hydrodistilate; Orange peel+C+P+B: bacteria incubated in presence of cellulase and pectinase orange peel pretreated hydrodistilate.

The bacterial growth rate of control sample tripled the bacterial density during 2h from 0.347 OD600nm to 1.062 OD600nm, confirming the viability of the bacteria. A control bacterium in growth AP medium was used to ensure that the bacteria developed normally and a false positive response on growth inhibition was eliminated (table 1).
The bacterial growth incubated in a presence of orange peel without enzymatic pretreatment, measured after a period of 2 hours, showed a significant increase in bacterial growth. Initial optical density was approximately 0.33 OD600nm and increased over 1.17 OD600nm after 2 hours (figure 1, the orange line). The linear curve, given by the equation y=0.4225x−0.131, indicates a steady increase in bacterial growth.
The bacterial growth in presence of cellulase (C) pretreated orange peel hydrodistillate showed a much lower growth pattern (figure 1, gray line) compared to bacteria incubated only with orange peel hydrodistillate. Overall, the bacterial growth remains low over the 2 hours, starting at about 0.32 OD600nm and slightly increasing to around 0.48 OD600nm. This suggests that the presence of cellulose-treated orange peel could release some compounds that have an inhibitory effect on bacterial strain. This bacterial growth inhibitory pattern also happened when the bacterial strain was incubated in presence of pectinase (P) pretreated orange peel hydrodistillate (figure 1, yellow line) or when bacteria was incubated in presence of orange peel pretreated with an enzyme mixt form by C and P (figure 1, blue line).

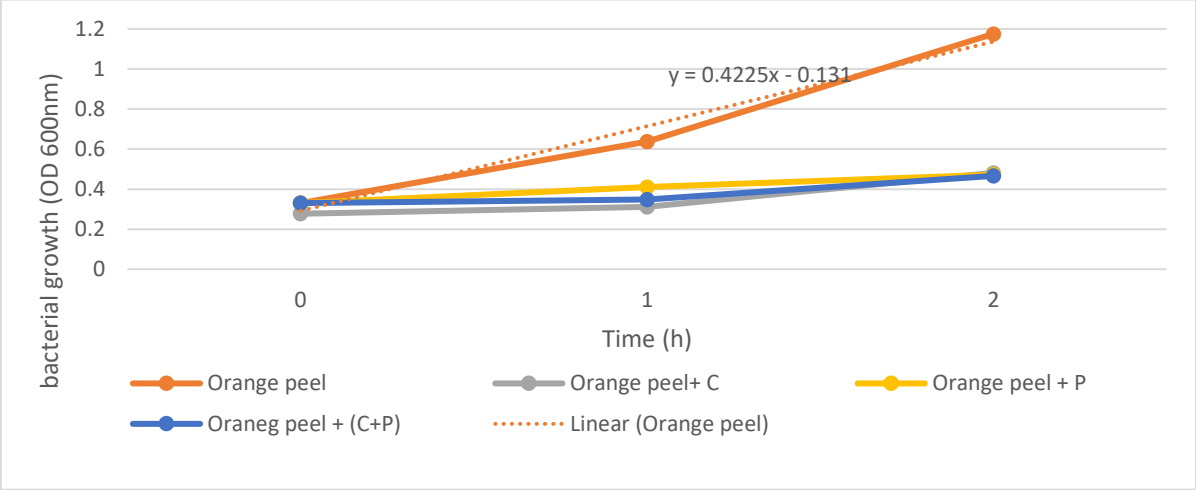

Fig. 1. Bacterial growth for the sample of orange peel extract with enzymatic pretreatment

The formula for calculating bacterial inhibition is often expressed as percent inhibition, and this is calculated using the equation (1).

$$\text{Bacterial inhibition (\%)} = 100-(E/\text{Control})*100 \quad (1)$$

where: Control is bacterial growth in AP medium; E is bacterial growth in the presence of the extract.

The link between bacterial inhibition and the antimicrobial effect of a vegetal extract lies in the extract's ability to suppress the growth and proliferation of bacteria. When a vegetal extract exhibits antimicrobial properties, it means that the bioactive compounds within the extract can interfere with various bacterial processes, leading to bacterial inhibition. This inhibition can manifest as a reduction in bacterial colony numbers, impaired bacterial growth, or the complete eradication of bacterial cells. Therefore, observing bacterial inhibition is a direct indicator of the antimicrobial effectiveness of the vegetal extract.
In the present study, the bacterial inhibition identified at the T1h measurement indicated a slight inhibition in the samples that underwent enzymatic pretreatment. The inhibition was much more pronounced at the 2-hour measurement, T2h. Optical density measurement showed a 50% inhibition for all three enzymatically pretreated samples, as shown in table 1.

GC-MS analysis

Chromatographic spectra of bioactive compounds found in mixed enzymatic pretreatment orange peel hydrodistillate showed the presence of a wide range of compounds such as: β-Myrcene, limonene, linalool, camphor, terpineol, carveol (figure 2). The analysis and interpretation of the spectral data were performed using the NIST MS Search 2.2 Library.

The most interesting compounds identified belonged to the classes of terpenes and terpenoids, which are described to have significant biological activities, according to the literature data [18÷31]. In table 2 are presented the most important terpenes and terpenoids identified and data regarding the main biological activities according to the literature. Pyrimethanil and isophorone have also been identified, indicating that the plants have received insecticide and pesticide treatments (table 2).

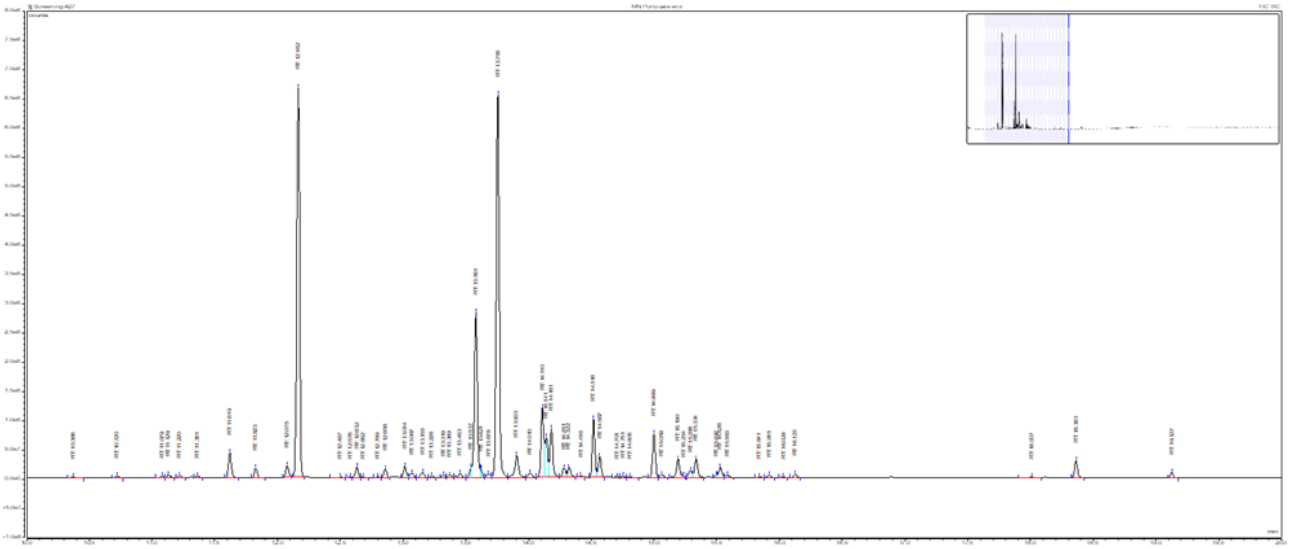

Fig. 2 Orange peel extract analyzed by gas chromatography-mass spectrometer

Table 2. Centralization of the most important compounds identified in orange peel extract

Compound	Reported Biological Activities	References
	Terpenes	
β-Myrcene	Analgesic, sedative, antidiabetic, antioxidant, anti-inflammatory, antibacterial, anticancer	Surendran S. et al., 2021 [18]
Limonene	Antioxidant, anti-inflammatory, antidiabetic, anticarcinogenic, antiatherogenic, hypolipidemic, cardioprotective, antifibrotic, hepatoprotective, antigenotoxic, antistress	Anandakumar, P., et al., 2020 [19]
Linalool	Antimicrobial, anti-inflammatory, analgesic, antihyperalgesic	Peana, AT and Moretti, MDL, 2008 [20]
Camphor	Antiviral, antimicrobial, antitubercular, antifungal, antitumor, anticonvulsant, muscle relaxant	Shokova, EA et al., 2016 [21]
Carvone	Antibacterial, antifungal, antiparasitic, antineuraminidase, antioxidant, anti-inflammatory, anticarcinogenic	Bouyahya, A., 2021 [22]
Citral	Antibacterial, antifungal	Saddiq, AA. and Khayyat, SA, 2010 [23]
Perillaldehyde	Antioxidant, antifungal, anti-inflammatory, anticancer, antidepressant	Erhunmwunsee, F. et al., 2021 [24]
Carvacrol	Antibacterial, antioxidant, antiviral, antiproliferative, anti-inflammatory, analgesic	Suntres, Z. E. et al., 2015 [25]

Terpenoids		
Eucalyptol	Antioxidant, anti-inflammatory, antimicrobial	Seol, GH, and Kim, KY, 2016; Ivanov, M. et al., 2021 [26, 27]
Terpineol	Antioxidant, anti-inflammatory, antiproliferative, antimicrobial, analgesic	Sales, A. et al., 2020 [28]
Carveol	Antidiabetic, antihyperlipidemic, hepatoprotective	Ahmed, MS et al., 2020 [29]
Geraniol	Antimicrobial, antitumor, antioxidant, anti-inflammatory	Mączka, W. et al., 2020 [30]
Others		
Pyrimethanil	Insecticide	Saladin, G. et al., 2003[31]
Isophorone	Pesticide	Saladin, G. et al., 2003 [31]

The use of specific enzymes allows for selective extraction, optimizing the antimicrobial properties of the extracts. Furthermore, enzymatic pretreatment is an environmentally friendly method, reducing the need for harsh chemicals and high-energy processes. Enzyme-assisted extraction is an attractive alternative to conventional extraction techniques since it is a sustainable and eco-friendly technology. Many studies have shown its highly positive effects in improvement of the yield of different classes of bioactive metabolites (e.g., polyphenols, carotenoids, proteins, polysaccharides, terpenes, and lipids) for nutraceutical and pharmaceutical applications.
The enzymatic pretreatment improved the efficiency and effectiveness of vegetal extraction as antimicrobial compounds, providing more potent natural alternatives to synthetic antimicrobial agents. Exploring the potential uses of orange peel can lead to innovative products and applications, promoting research and development in fields such as biotechnology and green chemistry.

Future research directions
The high concentration of terpenes and terpenoids from orange peel extracts, pretreated enzymatically or not, are the baselines for further research directions aimed at developing or identifying new antimicrobial compounds. These compounds will be isolated and purified by various chromatographically techniques followed by structural analyses using Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR). Furthermore, every isolated and identified compound will be tested for its antimicrobial activity on pathogenic bacterial strains to detect their minimum bacterial inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The research should also assess the potential synergy between terpenes/ terpenoids and conventional antibiotics to combat bacterial resistance, using methods like checkerboard and time-kill assays. Pursuing these directions can lead to the development of effective and safe antimicrobial products based on terpenes and terpenoids, aiding in the fight against infections and reducing antimicrobial resistance.

CONCLUSIONS

Enzymatic pretreatment enhanced the vegetal extracts obtained by hydrodistillation of orange peel. The experiments showed that the orange peel extract without any enzymatic pretreatment did not significantly inhibit bacterial growth, while enzymatic pretreatments with cellulase, pectinase, or their combination (C+P) inhibited bacterial growth. These additional vegetal compounds had antimicrobial properties compared to extracts from enzymatically untreated orange peels. Enzymatic pretreatment using pectinase and cellulase effectively degraded the cell wall components of orange peel, enhancing cell wall permeability and facilitating the release of essential oils. This process resulted in a higher yield of vegetal extracts during hydrodistillation, increasing the availability of active antimicrobial compounds. The enzymes streamlined the extraction process by reducing mass transfer resistance, making it more efficient and less energy-intensive. Additionally, the pretreatment improved the purity and concentration of antimicrobial constituents in extracts, enhancing their efficacy.

In summary, utilizing orange peel to produce antimicrobial extracts was a multifaceted strategy offering environmental, economic, and health benefits. It supported sustainable practices, reduced waste, and generated valuable products that could enhance food safety, healthcare, and personal care.

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